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REVIEW

Organophosphorus pesticide determination in biological specimens: bioanalytical and toxicological aspects

Sofia Soares[1,2] · Tiago Rosado[1,2] · Mário Barroso[3] · Duarte Nuno Vieira[4] · Eugenia Gallardo[1,2]

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Abstract

Organophosphorus insecticides, such as parathion-ethyl, quinalphos, chlorpyrifos, chlorfenvinphos or diazinon, are still widely used for pest control on crops. These compounds are extremely toxic to humans, and, even though specific legislation exists that controls the use of these substances, the frequency of toxic and/or fatal events and the existing data suggest that they are still easily accessed and the knowledge associated to the risks is not well-recognized. For these reasons, the determination of the exposure to these compounds, their detection (and of their metabolites as well) in biological samples, is of great importance in clinical and forensic toxicology, and, therefore, the development of techniques for this evaluation is an important task for laboratories. Most confirmatory analyses use blood, serum, plasma and urine as biological samples and are performed by either gas chromatographic-mass spectrometric or liquid chromatographic-mass spectrometric instrumentation, which represents the gold standard in what concerns high sensitivity. This paper will not only address the physical–chemical and toxicological aspects of this class of compounds but also perform a comprehensive and critical review on the analytical methods available for their determination in biological specimens, with special focus on the latest instrumental developments and sample preparation approaches.

Keywords Organophosphorus pesticides · Human biological samples · Bioanalytical methods · Forensic toxicological analysis

Introduction and history

Pesticides have always been used in its crude form from the earliest times, but their use as synthetic compounds happened early in the middle of the twentieth century [1].

Highly toxic organophosphorus compounds are a large group of organic phosphorus esters created in the 1930s before the World War II [2, 3], with the accidental discovery of tabun by Gerhard Schrader in 1937 [2, 4]. In addition to this finding, the starting point for the development of this class of pesticides was also the synthesis of alkyl phosphorofluoridates by Lange and von Krueger [5]. Consequently, investigations were initiated to develop other highly toxic compounds subsequently used as chemical warfare nerve agents, such as sarin, VX and soman [2, 4, 6, 7]. More recently, agents from the Novichok group are known to be weaponized, such as substance 33, A-230, A-232 (with Novichok-5 as binary analogue) and A-234 (with Novichok-7 as binary analogue). This family of compounds is related to the previously mentioned nerve agent VX; however, this group is considered to be 5–10 times more potent than the former [8].

After the war period, the use of these compounds and research on their development with the pesticide function increased [2, 9], due to the lower environmental stability, high toxicity and high efficiency [9, 10]. Consequently, these pesticides have become one of the most commonly used classes of substances in the world [11, 12].

These compounds have their importance and the beneficial effects associated with agriculture as agrochemicals used to minimize crop and post-harvest crop losses, ensuring the

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✉ Eugenia Gallardo
egallardo@fcsaude.ubi.pt

[1] Centro de Investigação em Ciências da Saúde (CICS-UBI), Universidade da Beira Interior, Av. Infante D. Henrique, 6201-556 Covilhã, Portugal

[2] Laboratório de Fármaco-Toxicologia—UBIMedical, Universidade da Beira Interior, Covilhã, Portugal

[3] Serviço de Química e Toxicologia Forenses, Instituto Nacional de Medicina Legal e Ciências Forenses, Delegação do Sul, Lisboa, Portugal

[4] Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal

effective production, and to control pests that affect them, as well as reducing the incidence of diseases transmitted by vectors [1, 10–13]. These compounds also have much shorter environmental half-lives compared to other classes of pesticides [14–16].

However, pesticides are manufactured to be toxic to living species and released into the environment; so, a large part of the population is exposed to these chemicals in the non-occupational environment or at the workplace, which makes it virtually impossible for exposure to be completely avoided. In addition, because of their widespread use and the fact that they are not specific to target organisms, they can be harmful to man, being considered an environmental health problem, which means that the associated risks (depending on whether the exposure is acute or chronic) should be properly evaluated [11, 12, 17–22].

Besides their reckless and indiscriminate use, the lack of use of safety devices during manufacture, storage, transport and agricultural application increases the risk of human exposure to these compounds, and as such a high number of accidental and intentional human intoxications occur [11, 23–29]. Furthermore, the widespread use of these compounds may increase insect resistance [12].

Although some of these insecticides have been restricted in 2001, their agricultural use is still important [30]. Though international organizations, such as the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations(FAO-UN) [31–33], have issued warnings and created strict legislation to prohibit and control the use of these compounds, they are still responsible worldwide for many deaths for accidental ingestion or accidental exposure through the skin and airways [33–36] and poisonings, either suicidal [37–39] or homicidal [40–43], each year [12, 13, 17, 23, 34, 44–48]. Most cases of suicides or attempts occur in rural areas, where people who use these pesticides usually store them at home, facilitating their ingestion [23, 49], since that they are still widely used in commercial and domestic agriculture or as chemical weapon [1, 17, 50, 51]. Cases of homicidal poisoning involving these compounds are less frequent due to the unpleasant odor and taste they present [23]. There is an estimation of 3 million accidental poisoning cases per year, an incidence of 220,000 deaths worldwide according to the WHO [36, 52] and more than 250,000 deaths per year from suicidal poisoning [13, 53–56]. A recent study involving several countries reports that the annual incidence of poisonings among agricultural workers varies from 3 to 10% per country [57, 58]. However, and despite these data, there is little published case reports in the literature [58–70].

The morbidity and mortality percentage due to intoxication by these insecticides is high and varies from country to country. Several factors contribute to this rate, such as the level of socioeconomic development, accessibility to these chemicals, importance of the local agricultural sector, as well as the delay in diagnosis and inadequate treatment. This reinforces the importance of toxicological screening for the correct diagnosis, since accurate information on the involved substance(s) is often rare. There is also a wide variety of compounds that may eventually be present, making it necessary to develop powerful and versatile analytical methodologies for their identification [62, 71].

The risk and the potentially increasing threat of terrorist attacks using either these pesticides or the above-mentioned nerve agents continue to be addressed today. This situation encourages the Chemical Weapons Convention (CWC) to ban their development, production and storage, which entered into force in April 1997. Recent episodes and intoxication statistics also reinforce the importance of toxicological aspects [72].

Structure and classification

Organophosphorus pesticides are a large group of highly toxic organic phosphorus esters, presenting the following basic structure (Fig. 1) (proposed by Gerhard Schrader in 1937 [2]).

All compounds are derived from the phosphoric acid molecule (P〓O) or from the phosphorothioic acid molecule (P〓S). The presence of one of these groups is important for the determination of human toxicity arising from these compounds [32, 73]. The thiophosphates have to be metabolized to the respective oxon, to become effective acetylcholinesterase inhibitors [9, 74], a cytochrome P450-mediated reaction that is susceptible to interindividual variations and consequently individual susceptibility to pesticides with this bond [75, 76].

The combination of the possible residues in the R and R′ positions and the different groups in the X position allows the synthesis of an indeterminate number of final derivatives. The R and R′ radicals may be alkyl, alkoxy, aryloxy or others, while X may be a phenoxy, thiophenoxy, phosphate, carboxylate or other groups [9, 73, 77].

Therefore, these pesticides can be divided into different subclasses, including phosphates, phosphonates, thiophosphates, phosphoramidates and phosphinates, which originate quite different physicochemical and toxicological properties [4].

They are usually liquid and non-polar compounds, which means that most of them are poorly soluble in water and tend to dissolve in fat [78]. Many of these compounds evaporate at room temperature. However, their stability depends on the pH of the medium where they are, and they will decompose under strongly alkaline pH values or in the presence of humidity. These properties have an influence on the importance of the routes of entry of these compounds, as well as the conditions that can best destroy them [79, 80].

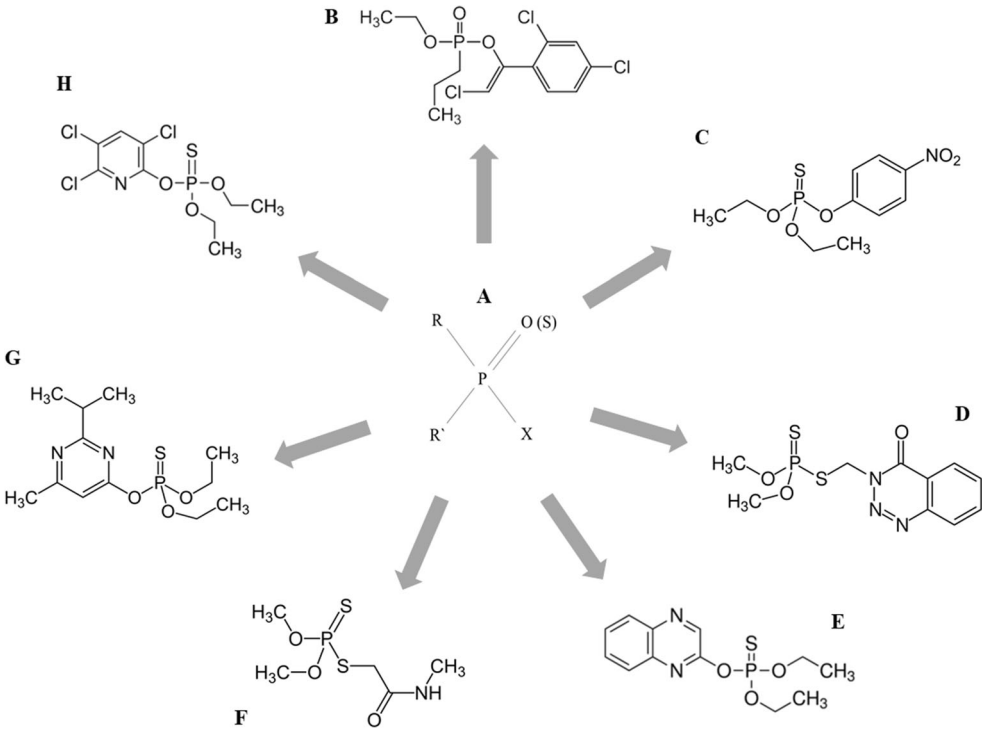

Fig. 1 Basic structure of organophosphorus insecticides (**a**) and examples of organophosphorus insecticides, which are (**b**) chlorfenvinphos, (**c**) parathion, (**d**) azinphos, (**e**) quinalphos, (**f**) dimethoate, (**g**) diazinon and (**h**) chlorpyrifos

Mechanisms of action

Organophosphorus pesticides affect the central nervous system, and nicotinic and muscarinic receptors are affected as well; these compounds are potent and irreversible inhibitors of cholinesterase activity (in whole blood), inhibit the activity of pseudocholinesterase (in serum) and inhibit the activity of acetylcholinesterase (in red blood cells), and this is known as the cholinergic syndrome [11, 13, 22, 54, 63, 81–84]. These effects are the cause of lethality associated to these insecticides. Other effects have also been reported, but they are not detailed because they are outside the scope of this review.

The toxicity of these pesticides is determined by this same inhibitory potency, by physicochemical properties, chemical and biological stability and through added additives. These compounds are commercialized as complex formulations and may therefore contain various organic solvents and emulsifiers, which may in turn increase their absorption and consequently toxicity. Intoxications can result in a broad spectrum of clinical signs depending on the intrinsic toxicity, the administered dose and the route of exposure [85–88].

The propagation of the action potential is due to the presence of acetylcholine followed by hydrolysis by the enzyme acetylcholinesterase. In a normal process, the enzyme hydrolyzes acetylcholine in two steps [89, 90]. Acetylcholine begins by binding to the active site of the enzyme, releasing the choline fraction and forming an acetylated version of it. Then, a water molecule attacks the enzyme releasing acetic acid plus the active enzyme, which is again available for a new catalytic cycle. In the case of organophosphorus pesticide poisoning, acetylcholinesterase is inhibited through a similar mechanism [89, 90]. The pesticide begins by phosphorylating the same active center that also binds to acetylcholine, but, in this situation, the phosphorus fraction is not released from the protein by hydrolysis [89, 90] and the phosphorylated enzyme is not capable of hydrolyzing its natural substract, losing its normal function in the catabolism of neurotransmitters [19]. Briefly, the main mechanism of toxic action is the covalent binding to the active site serine OH– group at the base of a deep gorge of the pivotal enzyme acetylcholinesterase. Inhibition of the physiological action of acetylcholinesterase to hydrolyze the neurotransmitter acetylcholine is due to its phosphylation, which includes both phosphorylation and phosphonylation [91].

With this, the altered hydrolysis of acetylcholine leads to its accumulation in the synaptic cleft, with consequent overstimulation of muscarinic and nicotinic receptors at the nerve–nerve and nerve–organs of the cholinergic system [22, 82, 92, 93]. This results in an eventual paralysis of nerves or muscles, which can produce neurotoxicity and result in death, which usually occurs following the impairment of respiratory muscles [23, 82, 94–96].

Moreover, it is known that this inhibition also involves, in addition to the central and vegetative nervous systems, the neuromuscular junctions with production of acute toxicity with high levels of acetylcholine acting on the above-mentioned receptors [22, 82, 92].

In addition to acetylcholinesterase, these compounds also exert a potent inhibitory effect over other serine esterases, namely butyrylcholinesterase and carboxylesterase [23, 97–99]. However, this inhibition does not result in additional acute toxic effects, but these enzymes can serve as endogenous source receptors for detoxification of a limited amount of incorporated compound [100, 101].

Consequently, the determination of the activity of the mentioned enzymes can be used in the biological monitoring of the exposure to those compounds that act on their inhibition [11, 54].

Acetylcholinesterase remains inhibited until a new enzyme is generated or until an enzyme reactivator (oxime) is directed. This phosphorylated enzyme can be reactivated by treatment with strong nucleophilic agents, such as those oximes referred previously, which reactivate acetylcholinesterase by removing the phosphoryl group. On the other hand, it may also undergo a spontaneous time-dependent process called "aging", where a dealkylation process occurs, leaving the enzyme irreversibly inhibited [19, 54, 102, 103].

In cases of intoxication by these pesticides, following the acute cholinergic crisis, the intermediate syndrome appears before the manifestation of delayed neuropathy. Symptoms such as respiratory paralysis, elevated serum creatine phosphokinase, weakness of proximal limb muscles, neck flexors and respiratory muscles and muscle fiber necrosis may occur in this syndrome. Studies have shown that this syndrome can occur from 24 to 96 h after organophosphate ingestion and after the individual recovers from acute cholinergic crisis. Additionally, delayed neuropathy induced by organophosphorus pesticides is a rare complication of acute exposure to some of these compounds, resulting from the phosphorylation and subsequent aging of at least 70% of neuropathy target esterase in peripheral nerves and consequent loss of activity [104, 105].

Toxicokinetics

Exposure to organophosphorus pesticides can occur through several routes, and this is important in the compound's rate of absorption into the systemic circulation. Thus, knowing the physicochemical properties of the compounds is essential for their toxicological evaluation [106].

The most common routes are inhalation, dermal contact and ingestion [22, 55, 82], with ocular exposure being a less common route of entry for these toxic compounds [107, 108].

After exposure to these compounds by vapor inhalation or intravenous administration, signs of intoxication (miosis, for instance) may appear early within a few minutes or hours, resulting in a rapid increase in their concentration in circulation resulting in acute toxicity [13, 22, 82, 92]. However, if poisoning occurs by percutaneous exposure to vapor or liquid, the signs may take hours to appear, and this results in a latency time between exposure and the onset of clinical symptoms, such as local sweating and fasciculations. There is also a delay in hours until they can be detected and quantified in blood. In the case of oral ingestion of these pesticides, gastrointestinal symptoms appear readily, and the risk is much more dominant, followed by the risk of contamination by inhalation [85, 106–121]. Systemic exposure through inhalation, dermal contact or ingestion may also lead to symptoms such as lack of vision and burning sensation [22, 82]. In addition to the acute toxicity associated to this exposure, long-term consequences of chronic toxicity may occur, such as infertility and cancer [20, 122–124]. Several studies suggest that there is an association between exposure to organophosphorus pesticides and reproductive disorders, because they affect the sperm structure and function and consequent deterioration of the semen, and any DNA damage may interfere with the transmission of genetic information during reproduction. These compounds may also alter reproductive function by reducing acetylcholinesterase activity in the brain, affecting pituitary gonadotropin. Both situations result in infertility [125, 126]. A compilation of papers by Mostafalou and Abdollahi [127, 128] shows that there is enough evidence demonstrating the role of exposure to organophosphorus pesticides in the incidence of diseases, such as cancer. The results show that the exposure to these compounds is related to cancers of the nervous system (brain tumor), haematopoietic system (lymphoma), digestive system (colorectal cancer), male (prostate cancer) and female (breast and ovarian cancer) reproductive systems and lung, thyroid and skin cancer. In addition, they concluded that carcinogenicity, via genetic or epigenetic mechanisms, is considered the most commonly reported toxic effect related to this class of pesticides, adding to endocrine disruption and oxidative stress. In addition, the organic solvents in which the pesticides are incorporated may also contribute to the mechanisms of toxicity.

These properties are an important factor to take into account for the development of appropriate methods for decontamination and drug treatment [129].

Organophosphorus pesticides easily cross alveolar and dermal membranes due to their lipophilic structures [130, 131], and as they are commonly applied as aerosols, inhalation and dermal exposures are the most common in accidental intoxications [130, 132]. However, the gastric mucosa also presents permeability to these compounds, being the classic choice in case of attempted suicide [131, 132].

Due to their chemical nature, these compounds have biological half-lives in the order of hours to a few days [133] and do not circulate in the bloodstream for longer time periods. These pesticides are distributed throughout the body, especially in adipose tissues [55], but, generally, do not accumulate due to their rapid biodegradation, being usually metabolized and excreted from the body within a few days [19].

After the distribution phase, the metabolism phase takes place. Some of these pesticides can be eliminated without

metabolism. However, as noted earlier, in most cases, rapid metabolism occurs, converting them to specific and non-specific metabolites [131, 134]. Most organophosphorus are activated by oxidation in the liver, and the enzymes involved in this process are the cytochrome P450 system and flavin-containing monooxigenases. The enzymatic systems involved in detoxification are phosphotriesterases (PTEs), carboxylesterases and glutathione-S transferases [135]. A major detoxification pathway is the hydrolysis by a type of esterases called PTEs, where the reaction products do not exhibit phosphorylation capability and each enzyme molecule is capable of deactivating many pesticide molecules [89, 90].

Nevertheless, these insecticides are generally metabolized into the more reactive form of oxon, which can bind to cholinesterases or be hydrolyzed into a dialkylphosphate (DAP) and a hydroxylated organic fraction specific of the pesticide. Alternatively, the intact pesticide may undergo hydrolysis prior to any conversion to the oxon form and the polar metabolites are excreted [19].

After metabolism, the metabolites may be excreted in faeces and expired air, although in low amounts; the main route of elimination is urine, specifically for the more polar metabolites [19, 131]. As an indication of exposure to these compounds, non-specific DAP metabolites may be measured in urine [136–142], and as this conversion does not originate specific metabolites, the absorbed pesticide cannot be identified [134]. Most of these compounds are excreted within 48 h as the parent pesticide, a mercapturate detoxification product, and as free or conjugated metabolites (glucuronides, sulphates) [143–150].

Case reports

Because of their widespread use in agriculture, organophosphorus pesticides represent a health problem worldwide and are involved in suicides, accidental self-poisonings and even homicides [25–29, 39, 43–48, 59–61, 68–70, 151]. In fact, a recent study reports that the annual incidence of poisonings among agricultural workers varies from 3 to 10% per country [57]. However, and despite these data, there is little published case reports in the literature. The following lines describe all case report cases found in the PubMed database using the following search strings (either alone or in combination): "organophosphorus pesticides" or "organophosphorus insecticides" and the different types of biological specimens.

An example of this is the case of a 79-year-old man found dead at home with a belt tied around his neck. A strong "chemical" odor was also detected. According to statements obtained, the deceased was being treated for prostate disease and depression, having already tried suicide twice. The autopsy findings included features such as edematous and emphysematous lungs, blood-like fluid from the parenchymal cut, presence of yellowish-white mucus in the bronchial tubes and trachea, congested liver, clear liquid with strong solvent odor, as well as walls with signs of erosive gastritis in the stomach and esophageal mucosa. Samples of blood and gastric contents were submitted for toxicological analyses. Negative results for drug and alcohol abuse were obtained. However, when the samples were analyzed by gas chromatography–tandem mass spectrometry (GC–MS/MS), diazinon was detected in blood at a concentration of 6.48 μg/mL [62].

In another case, a 43-year-old man attempted suicide by ingesting 100 mL of 5% fenitrothion and acephate emulsion. The patient was transferred to an emergency department, and blood samples were collected in dried heparin, treated and stored. On the next day, the samples were prepared and analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). The determined serum concentrations of fenitrothion and acephate were 4.5 and 7.2 μg/mL, respectively [63].

An 80-year-old man was found dead in bed, with no evidence of a struggle. A forensic autopsy was performed to elucidate the cause of death, where moderate rigidity was observed in all joints, chemical lesions on the right side of the face and upper portion of the body and congestive organs. The lungs were markedly edematous, the stomach contained a dark grey fluid that smelled strongly of organic solvents and the duodenum and intestine also contained a white milky liquid with the same odor. No drugs or ethanol were detected in the urine and blood samples, and the concentrations of methidathion in cardiac and peripheral blood were 66.2 and 8.33 μg/g, respectively. In the 30 g of stomach contents, the concentration of methidathion was 64 mg/g and that in the upper portion of the small intestine was 38 mg/g. The samples were analyzed by gas chromatography–mass spectrometry (GC–MS) [64].

Another paragon is that of a 24-year-old woman found dead in bed, with a strong smell of solvents or pesticides and faeces. There was a bottle whose label said it contained 200 mg/mL of chlorfenvinphos. Furthermore, it was said by the mother that it would be for the treatment of dog ticks. A suicide note was found, and the deceased had a history of depression, drug abuse and suicide attempts. At the autopsy, congested and edematous lungs were observed. Samples of cardiac blood, liver and stomach contents collected had a greenish-smelling colour with strong solvent odor and were analyzed by gas chromatography-flame ionization detector (GC-FID). Chlorfenvinphos was detected at concentrations of 8.6 mg/L in the cardiac blood, 60.0 mg/kg in the liver and 1.132 mg/L in the stomach contents [65].

An additional case is a 54-year-old man found dead on a football pitch. A bottle with a brownish fluid was found near the victim whose label mentioned "Denkavepon M50" and that it contained 47.5% of dichlorvos. The autopsy revealed diffuse

congestion of the internal organs and a haemorrhagic ulcer of the digestive tract. In the stomach, 150 mL of a volatile liquid was present. Samples of blood (cardiac and peripheral), urine, gastric contents, heart, lung, kidney and liver were collected and analyzed by GC–MS and liquid chromatography-photodiode array detector (HPLC-PDA). The concentration of dichlorvos in cardiac blood was 4.4 mg/L and that of peripheral blood was 1.3 mg/L. The pesticide was detected in heart tissue at 1400 mg/kg, and the kidney and lung concentrations were 1 and 2.1 mg/kg, respectively. In the urine, the concentration was 1.3 mg/L, and, in the stomach, it was 253 mg/mL, corresponding to 38 g dichlorvos [66].

Finally, the case of a 21-year-old woman, known to be a drug user, that was found dead at home. They found an empty syringe and a bottle of parathion-methyl. The blood sample was tested for the presence of alcohol, as well as for cannabinoids, opiates, cocaine, benzodiazepines, barbiturates and amphetamines by fluorescence polarization immunoassay. No evidence was found for the presence of drugs, and the blood alcohol concentration was 1.05 g/L. Organophosphorus pesticides were identified in the blood, but they were not detected in the liver, kidney and stomach contents. Parathion-methyl was determined in the blood at a concentration of 24 mg/mL using gas chromatography-nitrogen phosphorous detector (GC-NPD). According to the toxicological analysis, due to the absence of stomach contents, it was concluded that the cause of death was acute poisoning by intravenous injection of this organophosphorus [67].

Bioanalytical procedures for organophosphorus pesticide detection in biological specimens

In the field of clinical and forensic toxicology, it is essential to perform confirmatory tests for the diagnosis of acute or chronical poisoning situations. The choice of a type of analysis depends on the complexity of the biological specimens used for the detection and/or the determination of the toxic agents involved. The most commonly analyzed specimens are blood, plasma, serum and urine. However, it is also possible to use less conventional matrices, such as oral fluid, hair, sweat, tissues, vitreous humor and bile, among others. These alternative specimens are usually characterized by their complexity, and the toxic substances are usually present at low concentrations; in addition, in some cases, the amount of sample available for analysis is relatively small. Furthermore, post-mortem specimens can endow some difficulties compared to those obtained in clinical scenarios, namely those resulting from autolytic/putrefactive changes.

The use of highly sensitive techniques is therefore necessary. Typically, the detection of toxic compounds in clinical and forensic settings begins with a screening test followed by confirmatory analyses. For this purpose, immunoassays are usually the first approach [152].

In the literature, there are also cases of application of immunoassay tests to this class of compounds. An example is the work by Zhang et al. [153], in which a portable immunochromatographic strip-based biosensor was developed for the detection of trichloropyridinol (specific biomarker of exposure to chlorpyrifos) in saliva samples, which was successful in the direct analysis of complicated authentic samples. This immunosensor is based on gold nanoparticles, which are augmented in situations of reduced levels of analytes. These nanoparticles, when captured, can be observed without any equipment and quantified by a colourimetric reader. This biosensor exhibits a linear range and detection limit of 0.625–20 and 0.47 ng/mL, respectively.

Eskandari and Naderi-Darehshori [154] synthesized nanoparticles of a hydrophobic magnetic polymer (poly(styrene-divinylbenzene)) and studied their adsorption potential in order to determine trace levels of fenitrothion in both biological and environmental samples. The magnetite nanoparticles are added to the sample solution as a way of preparing a strong local magnetic field, which leads to a faster and more efficient precipitation of the nanoparticles from the extraction mixture. The method has a spectrophotometric determination at 571 nm and has been successfully applied to various samples, including plasma and urine. Linearity was observed in a range of 2–230 ng/mL of the compound, with relative standard deviations of 0.9–5.1% and recoveries in the range of 97.2–100.0%.

Lu et al. [155] have carried out a study in which the occupational exposure to diazinon by workers from Nicaraguan plantations, as well as their children, was evaluated by repeated sample collection over several days and biomonitoring using saliva samples. An enzyme-linked immunosorbant assay (ELISA) was used, and a significant correlation was found between the concentrations of the compound in saliva and plasma samples collected at the same time. Regarding children, this compound was not detected in most saliva samples, which was confirmed by urinalysis. Consequently, it has been suggested that saliva may be used to evaluate human exposure to this compound.

Furthermore, the work of Curwin et al. [156], where a comparison of an ELISA immunoassay and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analytical methods for measuring chlorpyrifos metabolite (3,5,6-trichloropyridinol (TCP)) in urine samples from Iowa farmers and non-farmers was presented. For this comparison, different statistical methods were used, and the analytical methods were moderately correlated (0.40–0.49), but the immunoassay method consistently presented significantly higher geometric mean (GM) estimates. This estimate of GM for TCP by immunoassay and LC–MS/MS varied between 14 and 14 and 2.9–3.0 μg/L, respectively. The limits of detection

(LOD) values for this metabolite were 0.50 and 3.32 μg/L by LC–MS/MS and ELISA, respectively.

Another approach to perform a screening in order to detect pesticides is the use of optical sensors. Yan et al. [157] carried out a recent review concerning this matter. In this review, new trends in high sensitivity optical sensors and their advantages have been compiled for the detection of pesticides, in particular the class of organophosphorus insecticides, which continues to be a concern because of their residues. Recent developments in optical sensors highlighted fluorescence detection (FL) strategies, such as enzyme-mediated methods, antibody-assisted methods, molecularly imprinted polymer (MIP)-based methods and aptamer-based and host–guest interaction-based sensors. Another category of sensors is shown by colourimetric (CL)-sensing strategy based on enzymes, antibodies and aptamers. Surface-enhanced Raman scattering strategies (SERS) focused on sensors based on gold, silver and bimetallic nanomaterials. The last group comprehends surface plasmon resonance sensor (SPR) and chemiluminescence strategy. These sensors bring advantages, compared to traditional methods of analysis, with respect to the sampling approach which becomes easier, faster and less expensive, while maintaining sensitivity of detection. These optical sensors show good performance to quantify pesticide residues in complex environments and food matrices, especially for simplification and visualization design. Since the devices are miniaturized and wireless networking is used, pesticide recognition can be transformed into a digital signal measurable by portable devices, which makes detection feasible outside the laboratory environment with minimal user involvement, representing a new generation of analytical devices for real-time detection. However, the use of this optical sensor has scarce application in human biological specimens.

All positive results in screening tests require therefore confirmation by a different method, which must be at least as sensitive as the screening test, allowing results with higher levels of confidence. The most common confirmation methods involve liquid (LC) or gas chromatography (GC) coupled to different detectors, but, since these compounds are volatile and of non-polar nature, GC is the most used instrumentation for analysis. Obviously, before running any chromatographic assay, the previous step of sample preparation is undoubtedly a fundamental step. This process is mandatory taking into account the complexity of the samples, the presence of interferences and in some cases the low concentrations of the compounds of interest. On the other hand, the physicochemical properties of the analytes have to be taken into consideration, since it is a quite heterogeneous group of compounds, being commercialized under different forms as well. The extraction methods are in constant evolution in parallel with instrumental techniques, thus allowing to reduce the complexity of sample treatment, and increasing sensitivity, precision and accuracy of the analysis. Once there are no reviews on the sample preparation techniques used to determine these compounds and considering that the greater volume of laboratory work is related to sample preparation, we have conducted a critical review of the approaches and recent trends available in the laboratories for the detection and quantification of these analytes in biological matrices, in order to aid analysts, particularly in the fields of clinical and forensic toxicology. Afterwards, the analytical aspects associated to the determination of these compounds will be discussed.

In order to simplify this information and facilitate the understanding and the readability of the paper, the existing literature on the matter was compiled in three tables, according to the sample preparation technique employed.

Literature search was performed using the PubMed database, and the search strings were “organophosphorus pesticides” or “organophosphorus insecticides” in the different types of human biological specimens. The most commonly used samples are blood, urine, plasma and serum, and papers since 2000 have been selected. For the remaining specimens, considered as alternative samples, papers from previous years were selected as well. All articles were screened independently by three of the authors to determine their relevance in the framework of the current review, and only papers selected by at least two of them were included.

Among the compilation made, articles in which more compounds were analyzed at the same time should be emphasized, such as the works of Kumari et al. [158], Lacassie et al. [159], Musshoff et al. [84], Luzardo et al. [62], Kudo et al. [160], Tarbah et al. [161], Russo et al. [162], Roca et al. [163] and Duca et al. [164]. In these works, analytical methods have been developed for the identification and/or quantification of a large number of organophosphorus compounds and some of their metabolites as well.

In Table 1 (Supplementary material), all the papers involving liquid–liquid extraction (LLE) and protein precipitation as sample preparation techniques for the analysis of organophosphorus pesticides are compiled. LLE is the most used technique for this class of compounds, while GC coupled to mass spectrometry is the most used instrumentation. In LLE, it is usual to use mixtures of organic solvents, such as dichloromethane, ethyl acetate, acetone and hexane. Luzardo et al. [62] used LLE as extraction technique with a mixture of these same solvents (dichloromethane, ethyl acetate and acetone) for the determination of a large number of pesticides in 2 mL of blood samples, and they were able to obtain limits of quantification between 5 and 50 ng/mL and recoveries from 77 to 105%, using GC and LC instruments both coupled to tandem mass spectrometry (MS/MS). Using a mixture of dichloromethane, acetone and hexane, Araoud et al. [165] were able to determine five compounds in 2 mL of serum, obtaining limits of quantification between 5 and 10 ng/mL and recoveries of 56 to 93%, using for analysis a GC–MS apparatus. In general, in the case of urine samples, with the use of only one

Table 1 Bioanalytical procedures using microextraction techniques for the determination of organophosphorus pesticides in biological samples

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	Recovery (%)	Reference
Chlorfenvinphos Chlorpyrifos Diazinon Parathion-ethyl Quinalphos	0.05; blood	DBS (Whatman 903 protein saver snap)	GC–MS/MS (EI)	100;100 (Chlorpyrifos; Parathion-ethyl, Chlorfenvinphos) 50; 50 (Diazinon) 100; 250 (Quinalphos)	1.33–1.58 (Chlorfenvinphos) 2.53–3.50 (Chlorpyrifos) 3.83–5.11 (Diazinon) 0.92–1.20 (Parathion-ethyl) 6.35–11.98 (Quinalphos)	[188]
Azynphos-ethyl Diazinon Chlorpyrifos Chlorfenvinfos Parathion-ethyl Quinalphos	0.1; blood	MEPS (C18)	GC–MS/MS (EI)	2500; 2500 (Azynphos-ethyl) 500; 500 (Diazinon, Chlorpyrifos, Chlorfenvinfos, Parathion-ethyl, Quinalphos)	60.70–68.15 (Azynphos-ethyl) 57.76–77.84 (Diazinon) 59.38–68.28 (Chlorpyrifos) 63.98–73.75 (Chlorfenvinfos) 62.30–76.32 (Parathion-ethyl) 70.48–77.57 (Quinalphos)	[187]
Phorate Phosphomidon Parathion-methyl Malathion Chlorpyrifos-methyl Chlorpyriphos Ethion	1; plasma	Mini QuEChERS (3 mL of 2% acidified ethyl acetate and 0.4 g of magnesium sulfate) and SPE (50 mg of PSA)	GC–MS/MS	0.80; 2.65 (Phorate) 3.64; 12.00 (Phosphomidon) 0.97; 3.21(Parathion-methyl) 0.40; 1.30 (Malathion) 0.92; 3.06 (Chlorpyrifos-methyl) 2.35; 7.75 (Chlorpyriphos) 1.78; 5.86 (Ethion)	91–98 (Phorate) 90–105 (Phosphomidon) 90–101(Parathion-methyl) 86–106 (Malathion) 81–105 (Chlorpyrifos-methyl) 81–102 (Chlorpyriphos) 94–100 (Ethion)	[189]
Diazinon	10; urine	SA-DSPE (0.5 mL of ethanol (as disperser solvent) containing benzophenone (2% (*w*/*v*))	GC-FID	0.3; 2	97.5–99.3	[195]
Chlorpyrifos Chlorpyrifos-methyl Dichlorvos Dimethoate Fonofos Ethion Malathion Methidathion Monocrotophos Paraoxon-methyl Phorate Phorate Sulfone Phorate Sulfoxide Phosalone Pirimiphos-ethyl Pirimiphos-methyl Quinalphos Triazophos	0.1; blood	FA-DSDME	LC–MS/MS (ESI)	0.0009; − (Chlorpyrifos) 0.015; − (Chlorpyrifos-methyl) 0.002; − (Dichlorvos, Phorate Sulfoxide) 0.004; − (Dimethoate) 0.038; − (Fonofos) 0.023; − (Ethion) 0.043; − (Malathion) 0.034; − (Methidathion) 0.029; − (Monocrotophos) 0.031; − (Paraoxon-methyl) 0.041; − (Phorate, Pirimiphos-ethyl) 0.013; − (Phorate Sulfone) 0.009; − (Phosalone) 0.110; − (Pirimiphos-methyl) 0.007; 0.01 (Quinalphos) 0.122; 0.01 (Triazophos)	102–109 (Chlorpyrifos) 96–104 (Chlorpyrifos-methyl) 98–102 (Dichlorvos) 99–104 (Dimethoate) 88–95 (Fonofos) 96–102 (Ethion) 86–95 (Malathion) 96–103 (Methidathion) 93–96 (Monocrotophos) 88–96 (Paraoxon-methyl) 92–102 (Phorate) 97–106 (Phorate Sulfone) 101–107 (Phorate Sulfoxide) 95–104 (Phosalone) 94–104 (Pirimiphos-ethyl) 86–93 (Pirimiphos-methyl) 96–106 (Quinalphos) 86–96 (Triazophos)	[158]
Chlorpyrifos Chlorpyrifos-oxon	5; urine	SFODME (2-dodecanol)	GC–MS (EI)	0.0048; 0.0156 (Chlorpyrifos)	100.3 (Chlorpyrifos) 109.6 (Chlorpyrifos-oxon)	[196]

Table 1 (continued)

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	Recovery (%)	Reference
				0.0038; 0.0129 (Chlorpyrifos-oxon)		
3,5,6-Trichloro-2-pyridinol *p*-Nitrophenol 2-Diethylamino-6-methyl-4-pyrimidinol 2-Isopropyl-4-methyl-6-hydroxypyrimidine 3-Chloro-7-hydroxy-4-methylcoumarin (3-chloro-4-methylumbelliferone) 3-Methyl-4-nitrophenol 1,2,3-Benzotriazin-4-one Dimethoate Omethoate Acephate Methamidophos Malathion dicarboxylic acid Diethylphosphate Diethylthiophosphate Diethyldithiophosphate Dimethylphosphate Dimethylthiophosphate Dimethyldithiophosphate	5; urine	Hydrolysis with β-glucuronidase and QuEChERS (magnesium sulfate; sodium chloride; sodium citrate; disodium citrate sesquihydrate)	UHPLC-HRMS (ESI)	–; 0.8 (3,5,6-Trichloro-2-pyridinol; *p*-nitrophenol; Omethoate) –; 1.6 (2-Diethylamino-6-methyl-4-pyrimidinol; 2-isopropyl-4-methyl-6-hydroxypyrimidine; 3-methyl-4-nitrophenol; methamidophos; dimethylthiophosphate; dimethyldithiophosphate) –; 2 (3-Chloro-7-hydroxy-4-methylcoumarin) –; 50 (1,2,3-Benzotriazin-4-one) –; 3.2 (Dimethoate; malathion dicarboxylic acid; diethylthiophosphate; diethyldithiophosphate) –; 10 (Acephate; diethylphosphate; dimethylphosphate)	72–106 (3,5,6-Trichloro-2-pyridinol) 90–101 (*p*-nitrophenol) 90–110 (2-Diethylamino-6-methyl-4-pyrimidinol) 40–107 (2-Isopropyl-4-methyl-6-hydroxypyrimidine) 54–80 (3-Chloro-7-hydroxy-4-methylcoumarin) 69–92 (3-Methyl-4-nitrophenol) 98–104 (1,2,3-Benzotriazin-4-one) 101.119 (Dimethoate) 103–125 (Omethoate) 71–86 (Acephate) 61–118 (Methamidophos) 61–91 (Malathion dicarboxylic acid) 69–111 (Diethylphosphate) 99–117 (Diethylthiophosphate) 71–85 (Diethyldithiophosphate) 96–122 (Dimethylphosphate) 88–127 (Dimethylthiophosphate) 98–114 (Dimethyldithiophosphate)	[163]
Dichlorvos Methamidophos Dimethoate Parathion-methyl Malathion Parathion	300; hair 3; urine	Active magnetic metal-organic framework hybrid material (Fe_3O_4/MIL-101 composite) and magnetic SPE procedure	GC-NPD	1.20; 4 (Dichlorvos) 2.28; 8 (Methamidophos) 0.21; 1 (Dimethoate) 0.38; 1 (Parathion-methyl) 0.30; 1 (Malathion) 0.28; 1 (Parathion)	76.8–82.6 (Dichlorvos) 92.1–94.5 (Methamidophos) 88.2–89.8 (Dimethoate) 84.1–86.9 (Parathion-methyl) 78.1–88.8 (Malathion) 87.5–88.6 (Parathion) for hair samples; 74.9–82.3 (Dichlorvos) 79.8–89.5 (Methamidophos) 80.1–83.7 (Dimethoate) 90.6–91.7 (Parathion-methyl) 89.3–90.8 (Malathion) 88.1–92.1 (Parathion) for urine samples	[198]
Diethylthiophosphate Diethyldithiophosphate	1; urine	MISPE and derivatization with PFBBr	GC–MS (EI)	3.0; 10.0 for both compounds	28.9–29.7 (Diethylthiophosphate) 31.9–32.7 (Diethyldithiophosphate)	[197]
Diazinon Fenitrothion Malathion	50; hair	HF-SPME	HPLC-PDA	0.6200; 2.000 (Diazinon) 0.0074; 0.024 (Fenitrothion) 1.3000; 4.200 (Malathion)	83–92	[181]

Table 1 (continued)

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL) (in ng/g)	Recovery (%)	Reference
Disulfoton Disulfoton-sulfoxide Disulfoton-sulfone Demeton-S Demeton-S-sulfoxide Demeton-S-sulfone	0.5; blood and urine	QuEChERS (pre-packed Extraction: 6 g of magnesium sulfate and 1.5 g of sodium acetate; SPE sorbent: 25 mg of PSA, 25 mg of end capped octadecylsilane, and 150 mg of magnesium sulfate)	LC–MS/MS (ESI)	0.90; 5.0 (Disulfoton) 0.85; 5.0 (Disulfoton-sulfoxide) 1.15; 5.0 (Disulfoton-sulfone) 0.97; 5.0 (Demeton-S) 0.66; 5.0 (Demeton-S-sulfoxide) 0.92; 5.0 (Demeton-S-sulfone) for blood samples, 0.46; 5.0 (Disulfoton) 0.73; 5.0 (Disulfoton-sulfoxide) 0.79; 5.0 (Disulfoton-sulfone) 0.76; 5.0 (Demeton-S) 0.86; 5.0 (Demeton-S-sulfoxide) 1.05; 5.0 (Demeton-S-sulfone) for urine samples	104–111 (Disulfoton) 99–102 (Disulfoton-sulfoxide) 94–95 (Disulfoton-sulfone) 102–106 (Demeton-S) 96–98 (Demeton-S-sulfoxide) 95–99 (Demeton-S-sulfone) for blood samples; 99–106 (Disulfoton) 92–100 (Disulfoton-sulfoxide) 87–101 (Disulfoton-sulfone) 99–112 (Demeton-S) 102–109 (Demeton-S-sulfoxide) 100–102 (Demeton-S-sulfone) for urine samples	[190]
Chlorpyrifos	0.3; cord blood	Online SPE (Hypersil GOLD C8)	LC–MS/MS (ESI)	0.01; 0.1	97.2	[191]
Fenitrothion Malathion Phenthoate	0.2; serum and urine	Monolithic extraction (MonoSpin® C18)	GC–MS (EI)	100; 100 for both samples	12.7–20.0 (Fenitrothion) 39.7–49.5 (Malathion) 23.4–29.2 (Phenthoate) for serum samples; 6.4–9.4 (Fenitrothion) 7.2–9.0 (Malathion) 7.9–9.2 (Phenthoate) for urine samples	[192]
Fenitrothion	0.2; serum and urine	Monolithic extraction (MonoSpin® C18)	GC–MS (EI)	25; 50 for both samples	88.8–106.1 for serum samples; 51.3–90.1 for urine samples	[193]
Phorate Diazinon Parathion-methyl Fenthion Quinalphos	9; urine	Cloud point extraction coupled with microwave-assisted back-extraction (Triton X-114 andisooctane)	GC-FPD	0.07; 0.21 (Phorate, Fenthion, Quinalphos) 0.04; 0.12 (Diazinon) 0.08; 0.24 (Parathion-methyl)	85–107	[194]
Parathion	0.1; urine and blood	SPME (DI) (CW/DVB, 65-μm)	GC–MS (EI)	3; 10 for urine samples, 25; 50 for blood samples	21.9–45.6 for urine samples; 6.6–6.7 for blood samples	[183]
Dimethoate	0.1; urine and blood	SPME (DI) (CW/DVB, 65 μm)	GC–MS (EI)	50; 100 for urine samples, 200; 500 for blood samples	1.24 for urine samples; 0.50 for blood samples	[184]

Table 1 (continued)

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	Recovery (%)	Reference
Quinalphos	0.1; blood and urine	SPME (DI) (CW/DVB, 65 μm)	GC–MS (EI)	10; 50 for blood samples, 2; 10 for urine samples	12.5–16.9 for blood samples; 24.5–27.7 for urine samples	[185]
Diazinon Parathion-methyl Malathion Parathion	0.3; blood, plasma 1 g; kidney, liver and cerebrospinal fluid	HS-SPME (PA, 85 μm)	GC-NPD	3; 20 (Diazinon) 50; 100 (Parathion-methyl) 45; 100 (Malathion) 18; 50 (Parathion) for blood samples, 5; 20 (Diazinon) 40; 100 (Parathion-methyl) 45; 100 (Malathion) 16; 50 (Parathion) for plasma samples, 4; 20 (Diazinon) 35; 100 (Parathion-methyl) 50; 100 (Malathion) 12; 50 (Parathion) for kidney samples (in ng/g), 10; 20 (Diazinon) 55; 100 (Parathion-methyl) 50; 100 (Malathion) 25; 50 (Parathion) for liver samples (in ng/g), 2; 20 (Diazinon) 35; 100 (Parathion-methyl) 40; 100 (Malathion) 8; 50 (Parathion) for cerebrospinal fluid samples	1.7 (Diazinon) 0.24 (Parathion-methyl) 0.14 (Malathion) 0.67 (Parathion) for blood samples; 1.39 (Diazinon) 0.23 (Parathion-methyl) 0.14 (Malathion) 0.78 (Parathion) for plasma samples; 1.34 (Diazinon) 0.36 (Parathion-methyl) 0.10 (Malathion) 0.87 (Parathion) for kidney samples; 0.31 (Diazinon) 0.16 (Parathion-methyl) 0.11 (Malathion) 0.32 (Parathion) for liver samples; 2.5 (Diazinon) 0.35 (Parathion-methyl) 0.28 (Malathion) 1.25 (Parathion) for cerebrospinal fluid samples	[180]
Parathion-methyl	2; blood (LLE) 0.3; blood, stomach content, liver and kidney (HS-SPME)	LLE (petroleum ether), purification for TLC and HS-SPME (PA, 85 μm)	GC-NPD	50; 100 (for blood samples and SPME) No data for the remaining compounds	46 for blood samples; 53 for liver samples; 54 for kidney samples. No data for the remaining compounds	[67]
Dichlorvos	1; urine	SPMEM	GC–MS (EI)	–	–	[186]
Bromophos-ethyl, Bromophos-methyl, Chlorfenvinphos, Chlorpyriphos, Demethon-S-methylsulfon, Diazinon, Dichlorvos, Dicrotophos, Dimethoate, Disulfoton, Edifenphos, Fenitrothion, Fenthion, Malathion, Methidathion, Mevinphos, Monocrotophos,	0.5; blood	HS-SPME (PDMS, 100 μm)	GC–MS (EI)	0.04; − (Bromophos-ethyl) 0.05; − (Bromophos-methyl, Chlorfenvinphos, Malathion) 0.03; − (Chlorpyriphos, Parathion-methyl) 0.01; − (Diazinon, Disulfoton, Fenthion, Quinalphos) 0.10; − (Dichlorvos,Mevinphos) 0.30; –(Edifenphos) 0.20; − (Methidathion)	8.2 (Bromophos-ethyl) 9.7 (Bromophos-methyl) 1.7 (Chlorfenvinphos) 8.3 (Chlorpyriphos) 2.9 (Diazinon) 0.1 (Dichlorvos, Mevinphos) 3.8 (Disulfoton) 10.6 (Edifenphos) 2.7 (Fenthion) 6.6 (Malathion) 1.3 (Methidathion, Quinalphos) 4.7 (Parathion-ethyl) 19.6 (Parathion-methyl)	[84]

Table 1 (continued)

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	Recovery (%)	Reference
Omethoate, Parathion-ethyl, Parathion-methyl, Phosphamidon, Quinalphos				(in μg/g) No data for the remaining compounds	No data for the remaining compounds	
Fonofos Chlorpyrifos Ethion	0.5; blood	HS-SPME (PA, 85 μm)	GC–MS/MS (EI; ion trap)	0.4; 1 (Fonofos) 0.2; 1 (Chlorpyrifos) 0.7; 1 (Ethion)	–	[18]
Dimethoate Diazinon Parathion-methyl Fenitrothion Malathion Fenthion Chlorpyrifos Methidathion Phosmet Azinphos-methyl	5; urine 2; serum	SPE (C18) and LLME (dichloromethane)	GC-ECD/NPD	1.4; − (Dimethoate) 0.6; − (Diazinon, Fenitrothion) 1.5; − (Parathion-methyl) 0.5; − (Malathion) 0.8; − (Fenthion) 0.7; − (Chlorpyrifos) 0.9; − (Methidathion) 2.0; − (Phosmet) 1.7; − (Azinphos-methyl) for SPE and urine samples, 2.0; − (Dimethoate, Parathion-methyl, Malathion, Methidathion, Phosmet) 3.0; − (Diazinon) 0.7; − (Fenitrothion, Fenthion) 6.0; − (Azinphos-methyl) for LLME and urine samples, 1; − (Diazinon, Fenitrothion) 5; − (Parathion-methyl) 2; − (Malathion, Fenthion, Chlorpyrifos) 3; − (Methidathion) 10; − (Azinphos-methyl) for SPE and serum samples. No data for the remaining compounds	68–102 (Dimethoate); 96 (Diazinon); 96–103 (Parathion-methyl); 95–103 (Fenitrothion); 90–99 (Malathion); 96–99 (Fenthion); 97 (Chlorpyrifos); 94–108 (Methidathion); 83–109 (Phosmet); 96–107 (Azinphos-methyl) for SPE and urine samples; 72–93 (Dimethoate); 61–77 (Diazinon); 84 (Parathion-methyl); 86–87 (Fenitrothion); 93–94 (Malathion); 85–91 (Fenthion); 50–64 (Chlorpyrifos); 99–107 (Methidathion); 93–114 (Phosmet); 98–109 (Azinphos-methyl) for LLME and urine samples; 86–88 (Diazinon, Fenthion); 91–93 (Parathion-methyl); 92–95 (Fenitrothion); 69–75 (Malathion); 87 (Chlorpyrifos); 100–105 (Methidathion); 57–70 (Phosmet); 119–121 (Azinphos-methyl) for SPE and serum samples; 71–98 (Diazinon); 59–92 (Parathion-methyl); 45–78 (Fenitrothion); 53–75 (Malathion); 98 (Fenthion); 26 (Chlorpyrifos); 93–104 (Methidathion); 46–96 (Phosmet); 53–112 (Azinphos-methyl) for LLME and serum samples. No data for the remaining compounds	[209]
Diazinon Parathion-methyl Fenitrothion Malathion Fenthion Chlorpyrifos Methidathion	3; serum and urine (human serum diluted 50-fold and urine diluted 10-fold with ultra-pure water)	SPME (PDMS, 100 μm)	GC-FPD and GC–MS (EI)	1; − (Diazinon) 3; − (Parathion-methyl) 4; − (Fenitrothion, Malathion, Fenthion) 5; − (Chlorpyrifos)	104–115 (Diazinon) 92–111 (Parathion-methyl) 79–83 (Fenitrothion) 92–108 (Malathion) 60–65 (Fenthion) 50–69 (Chlorpyrifos)	[182]

Table 1 (continued)

Compounds	Sample volume (mL or mg); matrix	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	Recovery (%)	Reference
				15; – (Methidathion) for serum samples, 0.1; – (Diazinon)	145–165 (Methidathion) for serum samples; 82–110 (Diazinon)	
				0.3; – (Parathion-methyl, Fenitrothion, Chlorpyrifos)	116–140 (Parathion-methyl) 108–126 (Fenitrothion) 124–141 (Malathion) 84–95 (Fenthion)	
				0.4; – (Malathion)	76–92 (Chlorpyrifos)	
				0.2; – (Fenthion)	136–145 (Methidathion) for urine samples	
				20; – (Methidathion) for urine samples		

CW/DVB, Carbowax™/divinylbenzene; DI, desorption ionization; ECD, electron capture detector; EI, electron ionization; ESI, electrospray ionization; FA-DSDME, fast-agitated directly suspended droplet microextraction; FID, flame ionization detector; FPD, flame photometric detector; GC, gas chromatography; HF-SPME, hollow fiber solid-phase microextraction; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HS-SPME, headspace-solid-phase microextraction; LC, liquid chromatography; LLE, liquid–liquid extraction; LLME, liquid–liquid microextraction; LOD, limit of detection; LOQ, limit of quantitation; MEPS, microextraction by packed sorbent; MISPE, molecularly imprinted solid-phase extraction; MS, mass spectrometry; NPD, nitrogen phosphorous detector; PA, polyacrylate; PDA, photodiode array detector; PDMS, polydimethylsiloxane; PFBBr, pentafluorobenzyl bromide; PSA, primary–secondary amine; QuEChERS, quick, easy, cheap, effective, rugged and safe; SA-DSPE, solvent-assisted dispersive solid-phase extraction; SFODME, solidified floating organic drop microextraction; SPE, solid-phase extraction; SPME, solid-phase microextraction; SPMEM, solid-phase micro-extraction membrane; TLC, thin-layer chromatography; UHPLC, ultra-high-performance liquid chromatography

In the absence of the LOQ value, the lowest point of the calibration curve was considered

solvent in LLE extraction, the authors of several studies were able to obtain lower limits of detection and quantification for these compounds and metabolites, when compared to other biological samples. Spaan et al. [166], using hexane as the solvent for LLE and GC–MS/MS instrumentation in the determination of several metabolites in 2 mL of urine, obtained limits of quantification between 0.01 and 0.1 ng/mL, with good recoveries (91–115%). Okamura et al. [167], using only 0.5 mL of urine, used ethyl acetate as extraction solvent, achieving limits of quantification between 1 and 2 ng/mL for two of the organophosphorus metabolites and detection limits of 0.3 and 0.5 ng/mL, with recoveries of 68 to 118%, by GC–MS analysis. With 0.8 mL of sample and LC–MS/MS instrumentation, Jayatilaka et al. [168], using dichloromethane as extraction solvent, were able to reach limits of quantification between 0.004 and 0.009 ng/mL for four of these insecticides, and recoveries around 100% were obtained. Using 2 mL of urine and the same solvent (dichloromethane), Montesano et al. [169] were able to obtain for the same compounds limits of quantification of 0.250 ng/mL and recoveries between 52 and 63%, using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) analysis.

Acetonitrile is the most commonly used solvent for protein precipitation, and there are several studies in which this is the only approach for sample preparation, both in urine and serum samples. Reemtsma et al. [170] using acetonitrile as the precipitation solvent, obtained limits of quantification of 0.3 to 2.5 ng/mL and recoveries between 69 and 122% for metabolites in 3 mL of urine and using a LC–MS/MS device. On the other hand, Wu et al. [171], with gas chromatography-flame photometric detector (GC-FPD) and GC–MS analysis, obtained limits of quantification between 2 and 10 ng/mL, also for metabolites in 1 mL of urine, with recoveries between 62 and 98%. Additionally, Inoue et al. [63], using 0.2 mL of serum and LC–MS analysis, reached limits of quantification between 250 and 1250 ng/mL for a number of organophosphorus pesticides, with recoveries between 82 and 107%.

Nevertheless, the large volumes of organic solvents that are required are considered, nowadays, a pitfall, and, therefore, some authors try to keep these volumes to a minimum, however assuring good extraction efficiencies. On the other hand, these two pretreatment techniques do not have a sufficient degree of efficiency in what concerns the removal of interferences, which are capable of increasing the matrix effect and hinder the signal of the analytes, especially at low concentrations; in addition, detection systems must be cleaned and maintained more often due to the deposition of fatty acids.

Solid-phase extraction (SPE) is known for presenting good compatibility with high-throughput multi-residue analytical procedures, and great extraction efficiencies are usually associated to it. Furthermore, and when compared to the previous approaches, there is no doubt that its main advantage is

automation (at least to a certain degree) as well as the considerable decrease of interfering compounds. With this technique, many types of cartridges with different sorbents can be used.

In this case, the most commonly used cartridges are reversed-phase hydrophilic–lipophilic balance, C18 and in some cases aminopropyl and anion exchange cartridges. Davis et al. [172] used 96-well plate SPE with Oasis HLB reversed-phase sorbent for the extraction of organophosphorus pesticide metabolites from 1 mL of urine and analysis through LC–MS/MS. They achieved limits of quantification between 0.03 and 0.1 ng/mL and recoveries between 50 and 98%. Using Oasis HLB reversed-phase sorbent, Raposo et al. [49], using 0.5 mL of blood and GC–MS instrumentation, Park et al. [13] in 1 mL of the same specimen and instrument and Olsson et al. [173] in 2 mL of urine and using LC–MS/MS for analysis have obtained limits of quantification between 50 and 100 ng/mL, 130 and 170 ng/mL and 0.25 ng/mL, respectively. In addition, they obtained recoveries between 31 and 109%, 71 and 94% and 81 and 99%, correspondingly. Cequier et al. [174], using 0.3 mL of urine, in 96-well plate SPE with Strata-X-AW-functionalized polymeric sorbent and an ultra-performance liquid chromatography–mass spectrometry–quadrupole time-of-flight (UPLC-MS-QTOF) instrument, have obtained limits of quantification between 0.50 and 4 ng/mL and recoveries from 39 to 112%. On the other hand, with analysis through LC–MS/MS, Odetokun et al. [175] in 0.6 mL of urine, used 96-well plate SPE with a weak anion exchange cartridge and obtained limits of quantification of 0.125 ng/mL and recoveries around 100%. Ueyama et al. [176] also used the SPE extraction technique with Strata-X-AW sorbent and LC–MS/MS for the analysis, obtaining limits of quantification between 0.3 and 1.2 ng/mL and recoveries between 64 and 101% in 1 mL of urine. Pitarch et al. [177] used SPE with Bond Elut C18 sorbent and GC–MS/MS analysis in the determination of several organophosphorus from 1 mL of serum, obtaining limits of quantification between 0.4 and 9 ng/mL and recoveries between 71 and 102%. Procedures involving solid-phase extraction (SPE) as sample preparation technique are compiled in Table 2 (Supplementary material).

The classical techniques of LLE and SPE continue to be widely used in the extraction of these compounds in various biological samples. However, the LLE technique, although relatively simple and faster, has the disadvantage of retaining fatty acids, which are subsequently harmful for the chromatographic instrumentation. On the other hand, SPE is the most used extraction technique and has advantages such as assisted automation and versatility of C18 and reversed-phase columns that allow the extraction and subsequent detection and quantification of a wide range of compounds.

However, these last sample preparation procedures use considerable volumes of organic solvents. For this reason, there is a growing trend to use "greener" extraction procedures, namely fully automatic and/or miniaturized techniques, which provide new operational paradigms. The papers in which microextraction techniques were used for sample preparation should also be emphasized. This criterion was used taking into account the advantages of these techniques, particularly low volume of sample and organic solvents, minimization of solvent waste (with the consequent environmental advantages) and the possibility of reusing the extraction device [178, 179]. Examples of these new paradigms are the changes to classical techniques such as SPE, incorporation of process automation, the use of dried blood spots (or dried matrix spots), molecular-imprinted polymers (MIPs), solid-phase microextraction, liquid–liquid microextraction or QuEChERS. Papers dealing with these new challenges in sample preparation are summarized in Table 1, and the main characteristics of each of them are highlighted.

Example of the use of this miniaturized techniques in the determination of organophosphorus pesticides is the published article by Kumari et al. [158]. These authors used a technique of fast-agitated directly suspended droplet microextraction (FA-DSDME) for the determination of a large number of compounds in small aliquots of blood (0.1 mL). Hernández et al. [18] and Musshoff et al. [84] also for blood samples (0.5 mL) used the headspace-solid-phase microextraction (HS-SPME) technique with difference only in the fiber used. Tsoukali et al. in two different papers [67, 180] have used the same technique (HS-SPME), but with the use of different and unusual samples as blood, plasma, kidney, liver, cerebrospinal fluid and stomach contents (0.3 mL from 1 g of tissue homogenate and 0.3 mL of whole blood or plasma), using the same fiber and the same instrumentation (GC-NPD). Ebrahimi et al. [181] prepared 50 mg of hair samples by hollow fiber solid-phase microextraction (HF-SPME) and analyzed them by high-performance liquid chromatography-photodiode array detector (HPLC-PDA). López et al. [182] developed an analytical procedure for 3 mL of serum and urine samples using solid-phase microextraction (SPME) with a polydimethylsiloxane (PDMS) fiber. Yet, Gallardo et al. [183–185] used the same technique, however using Carbowax™/divinylbenzene (CW/DVB) fibers for the preparation of blood and urine samples, using smaller sample volumes (0.1 mL). In turn, Yang and Xie [186] used the solid-phase micro-extraction membrane (SPMEM) preparation technique for 1 mL of urine samples for the determination of dichlorvos. Recently, Santos et al. [187] published an article using a miniaturized version of SPE, microextraction in packed sorbent (MEPS). These authors were able to reach limits of detection of 500 ng/mL for diazinon, chlorpyrifos, chlorfenvinfos, parathion-ethyl and quinalphos using volumes of whole blood as low as 0.1 mL and achieving recoveries between 57 and 78%. Moreover, Soares et al. [188], using only 0.05 mL of whole blood sample, applied the dried blood

spots (DBS) sampling approach and, although the recoveries were between 1 and 12%, limits of quantification between 50 and 250 ng/mL were obtained.

Worth noting is the use of a mini QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method by Srivastava et al. [189] and regular QuEChERS by Usui et al. [190] and Roca et al. [163]. This approach for sample preparation shows advantages in the versatility of the used sample, in these cases, plasma, blood and urine, as well as versatility in the type of equipment used for the analysis, which in the described cases was GC–MS/MS, LC–MS and ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS), respectively. It is important to mention that this QuEChERS technique has been accepted by many pesticide residue analysts, namely in the agricultural field. However, some modifications to the original QuEChERS method had to be introduced to ensure efficient extraction of pH-dependent compounds, to minimize degradation of some compounds which are susceptible to extraction conditions using acids or bases and to expand the spectrum of matrices and applications covered [152]. Liao et al. [191] used 0.3 mL of cord blood, which is an uncommon sample, with an online SPE system. Saito et al. [192, 193] used monolithic extraction for 0.2 mL of serum and urine preparation in the analysis of four organophosphorus compounds which were afterwards determined by gas chromatography–mass spectrometry (GC–MS). Jia et al. [194] used an innovative technique of urine cloud-point extraction coupled to a microwave-assisted back-extraction for the determination of some of these compounds, however with the disadvantage of the need to use a large sample volume (9 mL). Recently, Aladaghlo et al. [195] used solvent-assisted-dispersive solid-phase extraction (SA-DSPE) technique for the determination of diazinon, however in large volumes of urine (10 mL). Pelit and Yengin [196] developed a method for the determination of chlorpyrifos and one of its metabolites with a solidified floating organic drop microextraction (SFODME) sample preparation technique, but also needing considerable amounts of urine sample (5 mL) to accomplish the analysis.

Santos et al. [197] opted for a different preparation technique, molecularly imprinted solid-phase extraction (MISPE), for the determination of two metabolites of organophosphorus compounds in 1 mL of urine, with the disadvantage of the need for derivatization. Russo et al. [162] have used a gel permeation chromatography for extraction of kidney and liver samples (2–3 g), obtaining good analytical results.

Zhang et al. [198] developed a technique of active magnetic metal-organic framework hybrid material and a magnetic SPE procedure for the preparation of 300 mg of hair samples and 3 mL of urine.

Regarding the used equipment, Pérez et al. [199] and Barr et al. [200] are highlighted by the use of gas chromatography–high-resolution mass spectrometry (GC-HRMS). Hernández et al. [201], without sample preparation, used only hydrolyzed urine, Sancho et al. [202] have used a coupled-column liquid chromatography combined with tandem mass spectrometry (LC–LC–MS/MS) and Oya et al. [203] used an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) instrument.

The use of QuEChERS as an extraction technique earns a special mention because it has passed from the application to food samples to be applied in the extraction of biological specimens. In addition, it allows the extraction of more complex samples, such as viscera, which is more difficult when the classic techniques are employed. The other techniques of microextraction require further studies to be transferred from development and optimization to the applicability in routine laboratories.

Concerning hair samples, the number of studies is very scarce. It should be emphasized that from a general perspective hair samples require a prior extraction from the hair matrix (the same happens with meconium), for which a methanolic pre-extraction is often used, as was done by Tsatsakis et al. [204] (in 200–500 mg), Kanavouras et al. [205] (in 100 mg) and Knipe et al. [206] (in 100 mg) for hair samples and by Whyatt and Barr [207] for 500 mg of meconium samples. Moreover, Duca et al. [164] analyzed a large number of compounds of this class in 50 mg of hair samples, comparing two classical techniques for sample extraction, SPE and LLE, and using two highly sensitive instruments, GC–MS/MS and LC–MS/MS.

Also, in general, GC equipment can be pointed out as the most used in the analysis of this class of compounds. A final important note is that for the analysis of the DAPs, it is always necessary to derivatize the extracts prior to analysis by GC, and the most used derivatizing agent is pentafluorobenzyl bromide (PFBBr).

As a curiosity, with the use of LC–MS/MS equipment with atmospheric pressure ionization source (API), most of these insecticides form an ion during ionization, the protonated or deprotonated molecular ion and sometimes an adduct ion (e.g., sodium or ammonium adduct). Sources of atmospheric pressure chemical ionization (APCI) may be used instead of electrospray ionization (ESI), because it is less prone to sodium adduct formation. There are compounds which have the potential to form sodium or ammonium adducts in the mode of positive ions, most of the cases, or acetate or formate adducts in the negative ion mode with an API source. This characteristic confers a lower capacity of confirmation of the compounds and decreases the abundance of the protonated or deprotonated molecular ion, and there is greater potential for formation of adduct with ESI than APCI. Mobile phases of methanol have a higher degree of adduct formation, compared to acetonitrile, with respect to sodium adducts. This formation can be reduced or suppressed by the addition of ammonium or hydrogen ions [208].

In what concerns chromatographic analysis, the most used instrumentation for this class of compounds is GC. Regarding chromatography coupled detection, tandem MS detectors are critical for a range of substances with trace concentrations. In addition, other novel detection systems, such as TOF and orbitrap, are important for quantifying low-concentrated analytes and for identifying metabolite structures.

Conclusions and future perspective

The use of pesticides has affected man and human societies worldwide since ancient times to the present day.

This exposure to pesticides, specifically to the organophosphorus class, has been documented through the analysis of biological samples, mainly blood, plasma, serum and urine specimens. These specimens are the most commonly used for analysis in clinical and forensic toxicology. However, it is thought that toxicological studies should not only depend on the analysis of these samples but may be complemented by the analysis of other non-conventional biological matrices, such as oral fluid, hair and nails. One of the advantages of using these samples, compared to the more traditional ones, is that their collection is less invasive, being easier and less uncomfortable for the patient. However, few or no studies and analytical methods exist for the identification of these compounds in these samples.

Over the last few years, most of the procedures for sample preparation involve micro approaches, and there have been developments in GC–MS and LC–MS technologies for the identification of these compounds, being accessible to most laboratories nowadays. These methods contributed to reduce the amount of sample used in the analysis as well as to obtain lower LODs and LOQs.

In the future, instruments are expected to become even more sensitive and accurate, and other analytical technologies can be used. Consequently, as the sensitivity of the analytical equipment increases, there will also be a trend to reduce sample volume, which may be decisive in the case of little matrix availability; this is also important from the analytical point of view, since matrix-borne interferences will affect analysis to a lesser extent.

In addition, more efficient sample cleaning procedures are being developed, which are less time consuming and less expensive and harmful to the environment, with the use of lower amounts of organic solvents.

Concluding, what is being more and more necessary are rapid, sensitive and specific approaches for analysis, which are also miniaturized and prone to automation. Analytical methodologies for the identification of these compounds in cases of ante- and post-mortem intoxication should be developed and validated before routine use, and the analytical data should be shared through different communication platforms to let the results be available quickly enough to contribute to the immediate attention of an intoxicated individual.

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Compliance with ethical standards

Conflict of interest and ethical standards The authors declare that they have no conflict of interest. The manuscript does not contain clinical studies or patient data.

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